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Individuals from North America, Australasia, and Africa Are Infected with Four Different Genotypes of Human Herpesvirus 8

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To study human herpesvirus 8 (HHV-8) transmission between individuals and in populations, we developed a system for genetic fingerprinting of HHV-8 strains based on variation in the HHV-8 K1, glycoprotein B (gB), and glycoprotein H (gH) genes. Using this system, we sequenced nearly the entire K1 gene (840 bp); two segments of the gB gene (open reading frame 8), totaling 813 bp; and a 702-bp segment of the gH gene (open reading frame 22) from blood and tissue samples obtained from 40 human immunodeficiency virus-infected and noninfected individuals, including those with Kaposi's sarcoma, primary effusion lymphoma, or Castleman's disease. The specimen collection was assembled from individuals living in diverse geographical locations, including the United States, Australia, New Zealand, Uganda, and Zambia. As reported by others, K1 was the most variable gene, with up to 16% variation at the nucleotide sequence level and up to 32% variation at the amino acid sequence level. Despite this extensive sequence variation, the K1 amino acid sequence contained 14 conserved cysteine sites, suggesting a conserved tertiary structure. gB and gH sequences were highly conserved, in most cases differing by $<0.6\%$ in pairwise comparisons. K1 was the most useful gene for strain discrimination, but the other genes enabled the discrimination of strains with identical K1 sequences. Individuals from diverse geographic locations were infected with four different HHV-8 genotypes; strains did not strictly segregate by continent of origin. The majority of HHV-8 strains from the United States and Europe were relatively closely related, whereas some strains identified from Uganda and Australia were phylogenetically distant. Genotype I strains were the most common and were found on three continents. Identical sequences were found in specimens obtained from different body sites and at different times from the same individual.

INTRODUCTION

Human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma-associated herpesvirus, is a recently identified human herpesvirus belonging to the genus *Rhadinovirus* of the gammaherpesvirus subfamily. HHV-8 DNA has been found in both human immunodeficiency virus (HIV)-positive and -negative individuals and in all clinical subtypes of Kaposi's sarcoma (KS), including acquired immune deficiency syndrome (AIDS)-epidemic KS, classic European/Mediterranean KS, African endemic KS, and iatrogenic immunosuppression KS (Chang *et al.,* 1994; Moore and Chang, 1995). HHV-8 DNA has also been detected in lymphoproliferative diseases, including multicentric Castleman's disease and primary effusion lymphoma [PEL, also known as body cavity-based lymphoma (BCBL)] (Cesarman *et al.,* 1995a; Soulier *et al.,* 1995).

As the first identified human rhadinovirus, the HHV-8 genome is phylogenetically more closely related to that of herpesvirus saimiri (HVS), a nonhuman oncogenic rhadinovirus, than to the genome of Epstein–Barr virus (EBV), a human gammaherpesvirus of the genus *Lymphocryptovirus* (Moore *et al.,* 1996). HVS and EBV genotyping is based on the presence or absence of particular alleles of several open reading frames (ORFs), some of which are involved in cell transformation. Three HVS genotypes (A, B, and C) have been identified based on sequence divergence at the left end of the unique segment of the genome, a region required for viral oncogenicity. This region is highly variable among subgroups and is related to the transforming phenotype, so strains of genotypes A and C are highly oncogenic and readily transform simian T lymphocytes *in vitro*, whereas genotype B strains do not. Furthermore, the first gene at the left end of the HVS strain 11 genotype A unique segment [saimiri transformation-associated protein (STP-A11)]

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and the first two ORFs in the corresponding region of the HVS strain 488 genotype C [tyrosine kinase-interacting protein (Tip) and STP-C488] are essential for transformation in cell culture and for lymphoma induction in common marmosets (Biesinger *et al.,* 1990; Jung *et al.,* 1991). Similarly, EBV has been classified into two genotypes, EBV types 1 and 2, that differ among their EBNA-1, EBNA-2, EBNA-3, and EBERs alleles, leading to differences in their ability to transform primary B lymphocytes (Sample *et al.,* 1990; Aitken *et al.,* 1994). K1 is the first ORF located at the left end of the unique segment of HHV-8 genome (Russo *et al.,* 1996), a position equivalent to the HVS transforming genes. Expression of the HHV-8 K1 gene in rodent fibroblasts and in a chimeric herpesvirus in which the STP oncogene of HVS was replaced with K1 resulted in growth transformation, demonstrating the transforming potential of the gene (Lee *et al.,* 1998).

Because of the common features of gammaherpesvirus genomic organization and the genotyping schemes used for HVS and EBV, we developed a system for HHV-8 strain differentiation for use in molecular epidemiological studies and used it to investigate DNA and encoded protein sequence variation in materials obtained from both HIV-positive and -negative individuals, with or without KS, from diverse geographical locations. The system is based on amplification and sequencing of four segments from three HHV-8 ORFs: K1, glycoprotein B (gB), and glycoprotein H (gH). K1 was included in the analysis for the reasons described above, and gB and gH were included because we hypothesized that genes encoding glycoproteins that are potential immune targets might be variable. We found that the high level of sequence variation in K1 allowed derivation of phylogenetic relationships that were much more highly resolved than those obtained from gB or gH sequences. Nevertheless, the gB and gH sequences allowed resolution of relationships when identical K1 sequences were obtained. Individuals from diverse geographical locations had different HHV-8 genotypes; the majority of HHV-8 strains from the United States and Europe were relatively closely related, whereas more distantly related strains were identified from Uganda and Australia. Identical sequences were found in specimens obtained from different body sites and at different times from the same individual.

RESULTS

Strains and sequences

The demographic characteristics of patients and specimens included in the primary phylogenetic analysis are summarized in Table 1. DNA sequences of 24 K1, 56 gBN (from the N-terminus of gB), 40 gBC (from the C-terminus of gB), and 42 gHM (middle region of gH) gene fragments were obtained from specimens from 40 individuals. Because DNA sequences from different body sites or dif-

TABLE 1

Patients and Specimens

Specimen ID	Countries	HIV	Diseases	Specimen
US 1	US	+	KS	Biopsy
US 2	US	+	KS	PBMCs and cultured biopsy
US 3	US	+	PGL	PBMC-mt
US 4	US	+	KS	Biopsies[e]
US 5	US	+	KS	Cultured biopsy
US 6	US	+	KS	Cultured biopsy
US 7	US	+	KS	Biopsy
US 8	US	+	KS	Biopsy
US 9	US	+	KS	PBMCs and cultured biopsy
US 10	US	+	KS	Cultured biopsy
US 11	US	+	KS	Biopsy
US 12	US	+	KS	PBMCs and cultured biopsy
US 13	US	+	KS	PBMCs and cultured biopsy
US 14	US	+	KS	Cultured biopsy
US 15	US	+	KS	PBMCs
US 16	US	+	KS	Cultured biopsy
US 17	US	+	KS	Cultured biopsy
US 18	US	−	KS	Cultured biopsy
US 19	US	+	KS	Cultured biopsy
US 20	US	−	Healthy	PBMCs
US 21	US	−	Lymphoma	Effusion and PBMCs
US 22	US	+	KS	Cultured biopsy
US 23	US	+	KS	Cultured biopsy
US 24*[a]	US	+	KS	Biopsy
Au 1	Australia	+	CD	Lymph node
Au 2	Australia	+	KS	PBMCs
Au 3	Australia	+	KS	PBMC-mt
Au 4	Australia	+	KS	PBMCs
Au 5	Australia	+	KS and PEL	Lymph node-ms
Au 6	Australia	+	Lymphoma	PBMCs
Au 7	Australia	+	KS	PBMCs
Au 8	Australia	−	KS	Lymph node
NZ	New Zealand	+	KS and PEL	Effusion
Ge*[b]	Germany	+	KS	Biopsy
Ug 1	Uganda	+	KS	PBMCs
Ug 2	Uganda	+		PBMCs
Ug 3	Uganda	+		PBMCs
Ug 4	Uganda	+	KS	PBMCs
Za 1	Zambia	+	KS	Biopsy
Za 2	Zambia	+	KS	Biopsy
bl	bcbl/US	+	PEL	Cell line
bl*[c]	bcbl/US	+	PEL	Cell line
bc	bc 1/US	+	PEL	Cell line
bc*[d]	bc 1/US	+	PEL	Cell line

Note. All strains characterized were obtained from males except Ug 1 and Za 1 (female). mt, multiple time points; ms, multiple sections; CD, Castleman's disease; KS, Kaposi's sarcoma; PEL, primary effusion lymphoma; PGL, persistent generalized lymphadenopathy. Specimens with an asterisk as part of their identifier are from published sources.

[a] Moore *et al.,* 1996.
[b] Neipel *et al.,* 1997.
[c] Lagunoff and Ganem, 1997.
[d] Russo *et al.,* 1996.
[e] Biopsies from both lesion and uninvolved skin.

ferent time points of the same individual were identical, a single sequence from each gene fragment from each individual was included in the phylogenetic analysis. In 13 patients with a variety of syndromes (12 HIV-positive and 1 HIV-negative), sequences were obtained for all four gene fragments. Sequences were obtained from at least two gene fragments derived from 35 individuals (Table 2). Previously published sequences from the BC-1 and BCBL-1 cell lines (Russo *et al.,* 1996; Lagunoff and Ganem, 1997) and two AIDS-KS lesions (Moore *et al.,* 1996; Neipel *et al.,* 1997) were included in the primary analysis. Additional K1 sequences (Kasolo *et al.,* 1998; Nicholas *et al.,* 1998, and Morand *et al.,* 1999; GenBank accession number AF042370) were included in the secondary analysis.

Highly variable K1 sequences

The sequence segment analyzed contained 12 bp of 5′ nontranslated sequence and 828 bp of coding sequence (276 amino acids including all except the C-terminal 13 amino acids). Among the 18 K1 segments sequenced, there was extensive variation in both nucleotide and amino acid sequences. Across the collection, there was variation at 240 (29%) of 840 nucleotides positions and 130 (47%) of 276 amino acid positions (Fig. 1). In pairwise comparisons, the maximum sequence differences between two strains were 16% at the nucleotide level and 32% at the amino acid level.

Two types of controls indicate that the observed variation is real and not the result of experimental error. First, nucleotide and amino acid sequences of K1 fragments obtained by direct sequencing of PCR amplification products from BC-1 and BCBL-1 cell lines that had been propagated for >1 year in our laboratory were identical to their published sequences, except for one nucleotide missing from the 5′ nontranslated region of the published BCBL-1 K1 sequence (Lagunoff and Ganem, 1997) relative to all the sequences we determined. Second, identical sequences were obtained when specimens were reanalyzed, beginning at the initial PCR step (not shown).

As shown in Fig. 1, K1 of most strains was conserved in the N-terminal region (amino acids 1–19, the predicted signal peptide sequence) and the C-terminal region (amino acids 227–276, a predicted hydrophobic transmembrane domain and a small intracellular domain), but was highly variable in the central region (amino acids 20–226, a predicted extracellular domain). Of the central region, amino acids 51–92 (variable region 1 or VR1) and amino acids 191–231 (VR2) were the most heterogenous. Ug 1 and Au 1 were exceptional in that they had amino acid sequence variation across the entire gene. Although the K1 sequence was highly variable, the 14 cystine residues and eight *N*-linked glycosylation sites were highly conserved (Fig. 1), implying a conserved protein tertiary structure. Deletions of two to five amino acids occurred in the sequences of US 1, 4, and 6; Au 2 and 8; and Ug 3. The position of the deletion in US 1, US 6, and Au 2 was equivalent to that found in the previously published German sequence (Neipel *et al.,* 1997).

TABLE 2

HHV-8 Strain Discrimination and Genotyping

Specimen ID	Sequence group				Subtype	Genotype
	K1	gBN	gBC	gHM		
Ug 1	a	a	a	a		IV
Au 1	b	a	b	h		III
US 1	f	c	g	g		II
Au 2	c	c	h	f		II
Ge*	d	c	i	f		II
US 6	e	c	g	–		II
NZ	–	c	h	f		II[a]
Au 5	–	c	h	–		II[a]
Ug 2	q	c	g	c	F	I
Ug 3	p	c	c	d	F	I
Ug 4	–	c	g	d	F[a]	I
US 7	o	c	j	–	E	I
US 8	n	c	g	–	D	I
bl	m	c	f	f	C	I
US 2	m	c	g	f	C	I
US 10	m	c	–	–	C	I
bl*	m	–	–	–	C	I
US 5	–	c	g	f	C[a]	I
US 11	–	c	g	f	C[a]	I
US 12	–	c	e	f	C[a]	I
bc	1	c	g	e	B	I
bc*	1	c	g	e	B	I
US 13	–	c	g	e	B[a]	I
Au 3	k	c	g	b	A	I
US 3	i	c	i	f	A	I
US 4	h	c	i	f	A	I
Au 8	g	c	i	f	A	I
US 9	j	c	i	–	A	I
Au 4	–	b	g	e		ND
US 14	–	b	g	e		ND
US 16	–	c	g	–		ND
Au 6	–	b	–	e		ND
Au 7	–	b	–	e		ND
US 17	–	b	–	e		ND
US 18	–	b	–	e		ND
US 19	–	b	–	e		ND
US 20	–	b	–	e		ND
US 21	–	b	–	e		ND
US 22	–	c	–	–		ND
US 23	–	–	g	–		ND
Za 1	–	–	g	–		ND
Za 2	–	–	g	–		ND
US 15	–	–	d	–		ND
US 24*	–	–	–	g		ND

Note. –, Sequence not available; ND, not determined.

[a] Provisional.

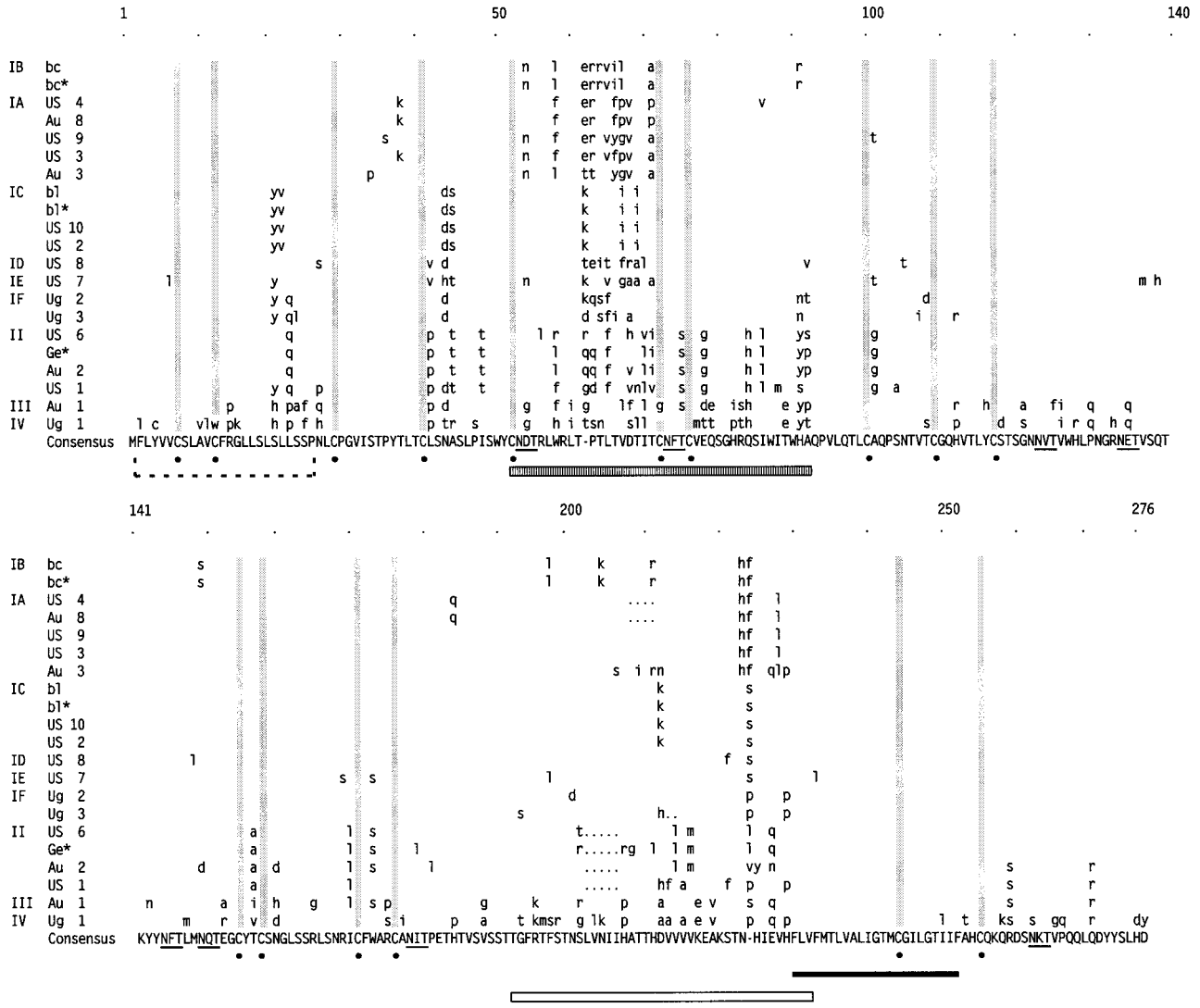

FIG. 1. Alignment of K1 amino acid sequences from a variety of DNA sources. The 276-amino-acid segments of K1 from the 21 strains included in the primary analyses are aligned. K1 genotyping is indicated on the left. The dotted line below the sequence indicates the predicted signal peptide; the heavy solid bar below the sequence indicates the predicted transmembrane domain; the bar filled with vertical lines and the open bar below the sequence indicate the variable regions, VR1 and VR2, respectively; the solid dots and gray vertical solid columns indicate cystine residues; and conserved potential *N*-linked glycosylation sites are underlined.

Conserved gBN, gBC, and gHM sequences

In contrast to K1, the gBN, gBC, and gHM sequences were highly conserved. The gBN and gHM segments were entirely within the gB and gH coding sequences. The gBC segment included 342 bp of C-terminal coding sequence and 129 bp of adjacent nontranslated DNA located downstream of the gB coding sequence and upstream of the DNA polymerase gene coding sequence (ORF 9).

At the nucleotide level, there was variation in three of 342 gBN positions, 30 of 471 gBC positions, and 14 of 701 gHM positions; the maximum difference in pairwise comparisons was 0.9, 5.7, and 1.1%, respectively. Within gBC, 20 of the 30 variable sites occurred only in the Ug 1 sequence; 20 variable sites, including 18 unique sites from Ug 1, clustered in a 25-bp segment of nontranslated DNA, located 41 bp upstream from the DNA polymerase gene start codon. At the amino acid level, variation occurred in 1 of 114 gBN positions, 2 of 114 gBC positions, and 9 of 234 gHM positions; the maximum sequence difference between two strains was 0.9, 1.7, and 2.6%, respectively. For all three loci, there was $<0.6\%$ nucleotide sequence variation in most pairwise comparisons.

Phylogenetic analysis

The quality of each phylogenetic reconstruction was evaluated by likelihood mapping analyses (Strimmer and von Haeseler, 1997) of aligned nucleotide sequences for

the K1, gBN, gBC, and gHM gene fragments and amino acid sequences for the concatenated K1 VR1 and VR2 region (not shown). In these analyses, the K1 DNA and K1 VR1VR2 amino acid sequence alignments resulted in a very high percentage (94 and 87%, respectively) of resolved quartets, which suggests that there is a true phylogenetic relationship among the sequences analyzed and that the data are very well suited for reconstructing of well-resolved trees. The gBN, gBC, and gHM sequence alignments gave high percentages of unresolved quartets; this is due to the low level of sequence variation in these genes. Because of this, the trees derived from these alignments have fewer indications of phylogeny than those derived from K1 sequences.

The phylogenetic inferences derived by quartet puzzling, a quartet maximum-likelihood method for reconstructing tree topologies, are shown in Figs. 2 and 3. All trees are unrooted. The K1 nucleotide sequences mainly clustered into two large clades (Fig. 2, top), designated genotypes I and II, whereas strains Au 1 and Ug 1 appear to represent two separate and less-populated clades, designated genotypes III and IV, respectively. Sequences within genotype I clustered into six subgroups, in which the sequences from Uganda strains (except for Ug 1) form one subtype (I-F); two American strains from KS lesions form subtypes I-D and I-E; BC-1- and BCBL-1-derived strains clustered into subtypes I-B and I-C, respectively; and five strains from North America and Australia are of subtype I-A. The phylogenetic tree derived by quartet puzzling from K1 nucleotide sequences has topology and branch lengths similar to those of trees derived from nucleic acid sequences by the maximum likelihood method using PHYLIP and from amino acid sequences using PUZZLE (not shown). The sequences representing the major branches of the tree (the four genotypes) are readily visualized on inspection of the amino acid alignment (Fig. 1). Furthermore, the tree derived from the amino acid sequences of the concatenated most variable regions of K1, VR1 and VR2, has the same topology as the tree derived from the K1 nucleotide sequences (Fig. 2, bottom). In trees derived from gBN, gBC, and gHM nucleotide sequences, the strains clustered into fewer groups than for K1; this is due to the lack of sequence variation among these fragments (Fig. 3).

Strain discrimination and genotyping of HHV-8

Table 2 summarizes our results relating to HHV-8 strain discrimination and genotyping. Sequence groups for each gene are defined based on the tree branching. Genotypes are designated according to the phylogenetic distances of K1 gene (Table 3) and the combination of main branch patterns of the phylogenetic trees derived from all four gene fragments but depend most heavily on the K1 tree because it is the best resolved. The subtypes are named according to either their sequence cluster in the tree derived from K1 sequences or provisionally, based on their sequence groups in trees derived from gBN, gBC, and gHM sequences when K1 sequence was not available. Based on the likelihood mapping analysis and the reconstructions of K1, gBN, gBC, and gHM phylogenies, the HHV-8 strains analyzed can be divided into four clades, designated genotypes I, II, III, and IV. As described above, the strains in genotype I can be further divided into subtypes A, B, C, D, E, and F. Some strain classifications, for example, Au 4, US 14, and Au 6, remain undetermined because their K1 sequence is not available and, at the same time, they have group b gBN sequences (Fig. 3), which are not seen in strains from genotype I–IV.

As shown in Figs. 2 and 3 and summarized in Table 2, 16 of 21 strains could be differentiated based on their K1 DNA sequence alone. However, for strains with the same K1 sequences, their gBN, gBC, and gHM sequences allowed them to be differentiated. For example, strains BCBL-1 (designated bI) and US 2 could be distinguished by their gBC differences. Moreover, the subtypes of certain strains not having K1 sequence available, such as NZ, Au 4, US 14, US 5, US 11, and US 12, were provisionally determined based on their gB and gH sequences.

Individuals from diverse geographical locations are infected with different genotypes of HHV-8

As shown in Table 3, the majority of HHV-8 strains from the United States were relatively closely related (genotypes I and II), whereas some strains identified from Uganda and Australia were phylogenetically distant (genotypes III and IV) (Figs. 2 and 3). Among the sequences analyzed, genotypes I (subtypes A, B, C, D, and E) and II were found in the United States; genotypes I (subtype A), II, and III were found in Australia and New Zealand; and genotypes I (subtype F) and IV were found in Uganda (Table 2).

After our primary analysis was completed, additional information regarding HHV-8 K1 sequences became available, including 12 Zambian strains from nine children with HIV-negative or -positive childhood endemic KS (KS-CE) and three febrile infants (FI), four Danish strains from adult AIDS KS (DM-KS) (Kasolo *et al.,* 1998), one strain from a cell line (BBG-1) derived from peripheral blood lymphocytes of a male French AIDS patient (Morand *et al.,* 1999), and seven strains sequenced by Nicholas *et al.* (1998) (five from the United States, one from Zaire, and one from Uganda). For the FI/KS-CE and DM-KS strains, the K1 sequences spanned 220 nucleotides (residues 24–243), encoding 73 amino acids; one FI sequence spanned 616 nucleotides (residues 64–679), encoding 205 amino acids. The French sequence in-

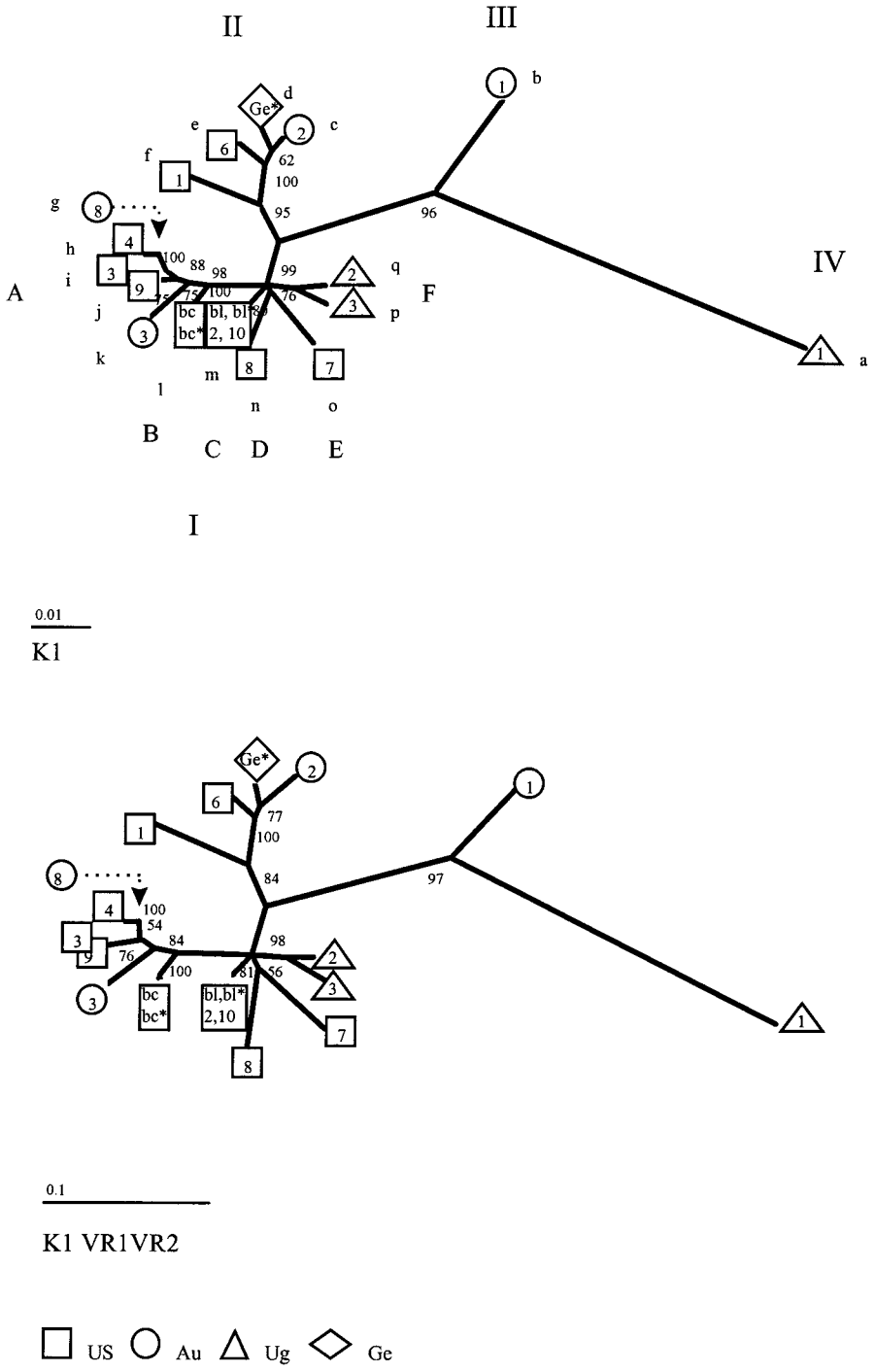

FIG. 2. K1 phylogenetic relationships; 840 bp of nucleotide sequences (top) and 71-residue concatenated and degapped VR1 and VR2 amino acid sequences (bottom) from the same set of 21 strains were used in these analyses (primary analyses). The trees were constructed by approximate maximum likelihood values (PUZZLE 4.0) with the HKY model of substitution and 1000 puzzling steps for nucleotide sequences and with the JTT model of substitution and 10,000 puzzling steps for amino acid sequences. The reliability values of quartet puzzling tree searches for the important internal branches are shown. The scale bar is at the bottom left of each panel. Strains are labeled alphanumerically, and countries of origin are indicated by different geometrical shapes. The sequence group (lowercase), subtype (uppercase), and genotype (Roman numerals) are also indicated in the top panel. All trees are unrooted. Arrows point to the nodes of tree branches. Country code: USA (US), Australia (Au), Germany (Ge), and Uganda (Ug).

cluded the full-length K1 gene, and the sequences published by Nicholas *et al.* (1998) included two short segments of amino acids (VR1 and VR2), with each segment containing 41 amino acids (residues 52–92 and 191–231, respectively). Likelihood mapping and phylogenetic analyses (secondary analyses) were performed with the in-

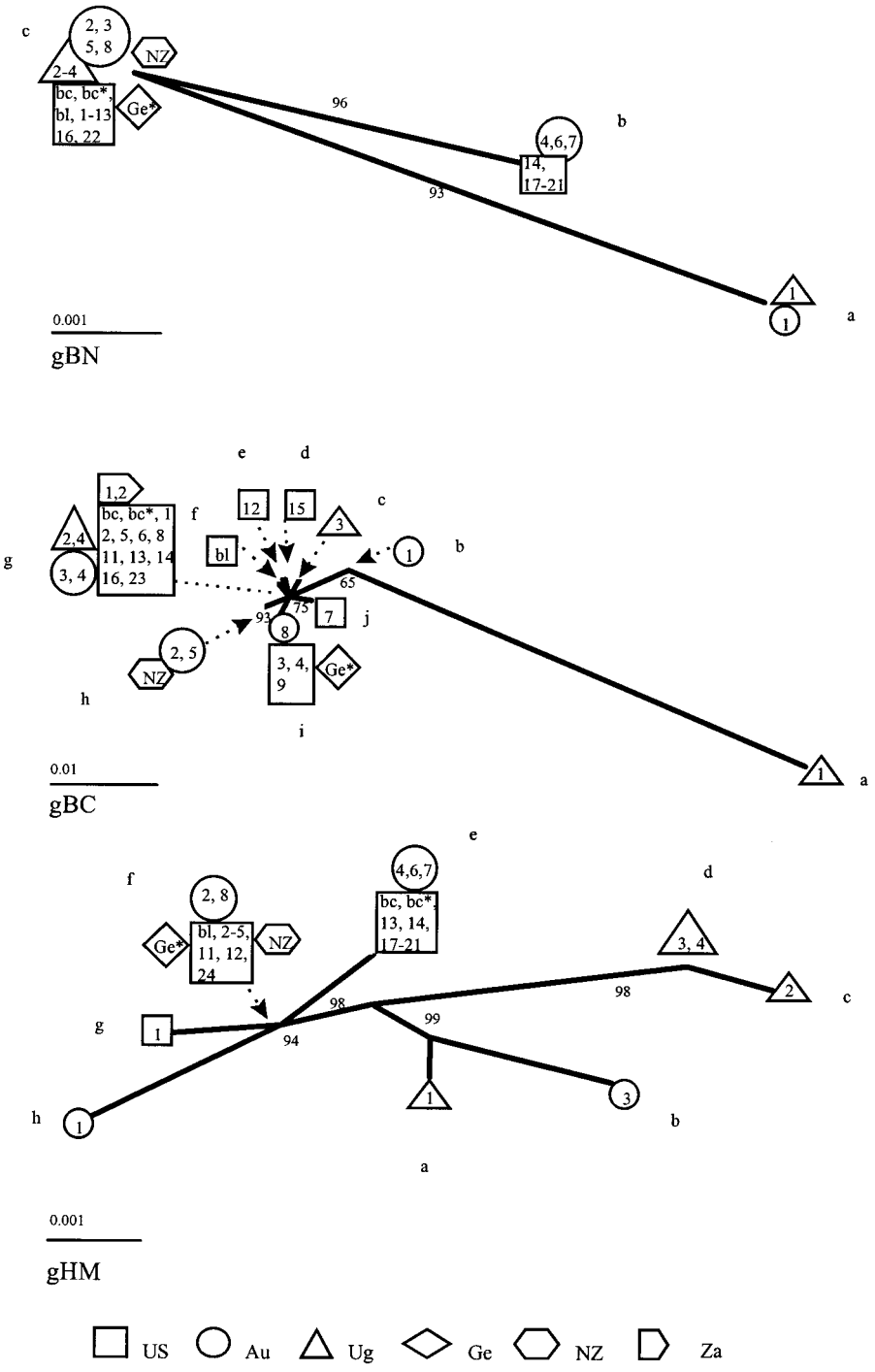

FIG. 3. Phylogenetic relationships among nucleotide sequences of HHV-8 gBN (38 sequences), gBC (33 sequences), and gHM (31 sequences). Trees were constructed by 10,000 puzzling steps. Sequence groups for each gene are indicated (lowercase) based on tree branching. In panel gBC, arrows point to the tips of branches except for sequence groups b and g, for which the arrows point to branch nodes. In panel gHM, the arrow points to a branch node. Country code: USA (US), Australia (Au), Uganda (Ug), Germany (Ge), New Zealand (NZ), and Zambia (Za). Other methods and notations are as described for Fig. 2.

clusion of these additional K1 sequences. To obtain the most information from the available sequences, several analyses were performed because all except one of the additional sequences were shorter than the segments we sequenced and had different amounts of overlapping information.

The most informative analysis was based on a 220-nucleotide K1 segment available from 38 strains, includ-

TABLE 3

Maximum Genotype-to-Genotype Distances for HHV-8 K1

Genotype	I (%) nt/aa	II (%) nt/aa	III (%) nt/aa	IV (%) nt/aa
I		7/15	10/22	16/32
II			10/20	15/31
III				13/24
IV				

Note. Uncorrected distance by pairwise comparisons. nt, nucleotide; aa, amino acid.

ing the 21 sequences used in our initial analysis, 16 from Kasolo *et al.* (1998), and 1 from France (Fig. 4). All of the additional European strains (4 Danish and 1 French) clustered into genotype II, where strains from Germany, United States, and Australia were previously classified based on the longer nucleotide sequences available from this study (Figs. 2 and 4). In addition, the 12 strains from Zambian children clustered into genotype I subtype F along with the two previously assigned Ugandan strains (Ug 2 and Ug 3) (Figs. 2 and 4). In a likelihood mapping analysis, this tree had a high percentage (87%) of resolved quartets, indicating that it reflects true phylogenetic relationships. Moreover, phylogenetic analysis based on the 73-amino-acid sequence encoded by this segment gave a tree with topology similar to that shown in Fig. 4 (not shown).

A 616-nucleotide segment encoding 205 amino acids was available from 23 strains, including the 21 sequences used in our initial analysis, 1 FI strain from Zambia, and the French strain. Groupings similar to those shown in Fig. 4 were obtained from both the nucleotide and amino acid sequences (not shown).

To incorporate the shorter VR1 and VR2 segments in the analysis, a phylogenetic analysis was performed with 71-residue concatenated and degapped K1 VR1 and VR2 amino acid sequences from a total of 29 strains, including the 21 sequences used in our initial analysis, the 7 previously reported by Nicholas *et al.* (1998), and 1 from France. In this analysis (not shown), the additional strain from Zaire (431KAP) fell into genotype IV, which was previously occupied only by a single Ugandan strain (Ug 1) (Fig. 2). In addition, 3 of the additional US strains fell into genotype I, and 2 fell into genotype II, as did the

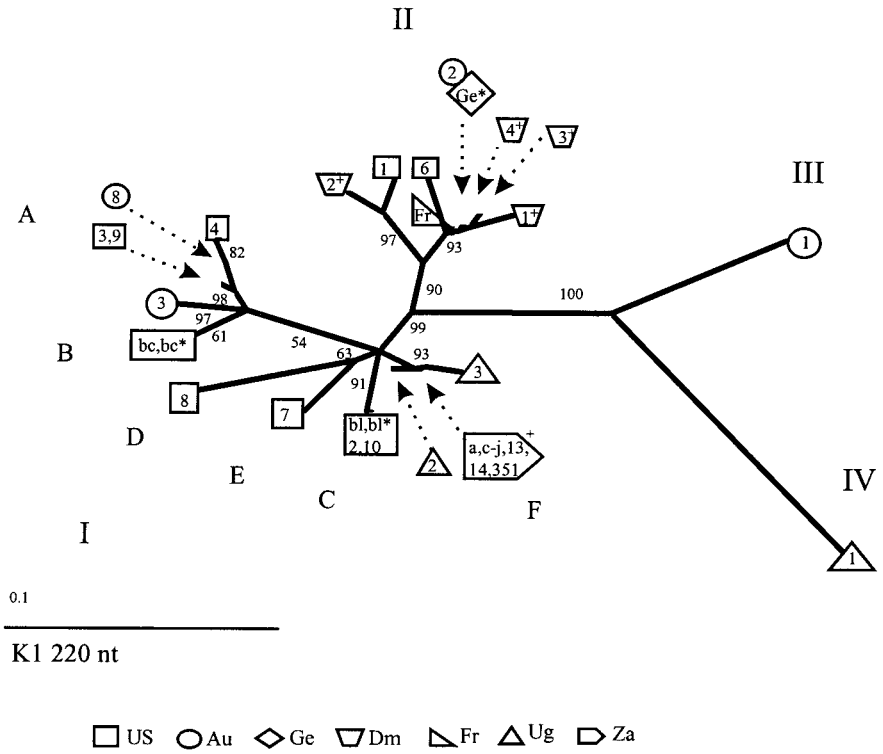

FIG. 4. K1 phylogenetic relationships based on 220 bp of nucleotide sequences from an expanded data set. 220-bp segments of nucleotide sequence from the 21 sequences used in the analysis shown in Fig. 2, 16 strains reported by Kasolo *et al.* (1998) (+), and 1 French strain (GenBank accession number AF042370) were used in this analysis (secondary analysis). The trees were constructed by 1000 puzzling steps. Strains are labeled alphanumerically, and countries of origin are indicated by different geometrical shapes. The subtype (uppercase) and genotype (Roman numerals) are indicated. All trees are unrooted. Arrows point to the tips of branches, except for strain Au 8, for which the arrow points to a branch node. Country code: USA (US), Australia (Au), Germany (Ge), Denmark (Dm), France (Fr), Uganda (Ug), and Zambia (Za). Other methods and notations are as described for Fig. 2.

French strain. The genotype of the one Ugandan strain reported by Nicholas *et al.* (ST1) could not be determined because it was distant from all of the genotypes identified in this study; more extensive sequencing will be required for its typing.

A 30-amino-acid segment that includes part of VR1 is common to all 45 strains. Unfortunately, the short length of the segment led to a large number of unresolved quartets (>30%), precluding meaningful tree reconstruction.

HHV-8 strains from different body sites in the same individual

Seven patients had either two or three specimens collected from different locations of the tumor (different sections) or more than one body site, such as KS skin lesion and peripheral blood mononuclear cells (PBMCs), effusion and PBMCs, or biopsies from KS skin lesion and uninvolved skin (Table 1). The K1, gB, and gH sequences derived from these specimens (US 2, 4, 9, 12, 13, and 21 and Au 5) were identical in multisite specimens from the same patient.

HHV-8 strains from different time points in the same individual

HHV-8-specific sequences were identical in PBMCs obtained at different time points from patients Au 3 and US 3. For patient US 3, this was true for nine PBMC specimens collected over a period of almost 9 years (Meng *et al.,* manuscript in preparation).

DISCUSSION

HHV-8 genotyping

The most rigorous viral genotyping is based on the analysis of complete genomic sequences, but given their genome sizes, this is not practical for herpesviruses. When subgenomic segments are used for genotyping, it is important that the segments analyzed are sufficiently variable from strain-to-strain to allow robust phylogenetic analysis. In addition, analysis is simplified if the variable region is stable in the infected individual, as was demonstrated here and discussed below. In this work, we studied sequence variation in three HHV-8 genes: K1, gB, and gH. K1 sequences were sufficiently variable to allow robust phylogenetic analysis, as assessed by likelihood mapping analysis, but the gB and gH sequences were highly conserved and did not allow for high-resolution comparisons. Nevertheless, the variation in these genes was sufficient to allow discrimination of the small number of strains with identical K1 sequences. It will be important to identify other markers that can reliably complement K1 for HHV-8 genotyping and strain discrimination and also allow identification of interstrain recombination events.

The typing scheme described here differs in several respects from systems described by others. Zong *et al.* (1997) proposed an HHV-8 typing scheme based on sequence variation in HHV-8 ORF26, ORF75, and the upstream region of ORF75. Within the sequences analyzed by this group, there was variation in only 38 of 2500 nucleotide positions (including silent nucleotide substitutions) across a collection of 12 specimens. The HHV-8 strains in their study segregated into three main groups (A, B, and C). Di Alberti and colleagues (1997) classified HHV-8 into four subtypes (A, B, C, and D) and a miscellaneous group (E) based only on the two nucleotide substitutions that led to predicted changes in amino acids in a 170-nucleotide segment of ORF 26. The limited sequence variation in the genomic segments analyzed in these studies led to difficulty in strain discrimination and lack of robustness in the phylogenetic inferences. For example, Kasolo *et al.* (1998) reported that strains identified from Zambian patients with childhood endemic KS or febrile illness were unclassifiable using the typing system of Zong *et al.* (1997).

By comparing the sequences of ORF 26, ORF 75, and ORF 22, the Zambian strains clustered into a new group and clearly did not conform to the A, B, or C types of Zong *et al.* (1997). Some Zambian strains had no diagnostic residues as defined by Zong *et al.* (1997), whereas others had mixtures of residues postulated by Zong *et al.* (1997) to be diagnostic for types A, B, or C. Moreover, all of the Zambian strains had additional diagnostic residues that did not conform to the A, B, or C grouping. Based on comparisons of a 220-bp segment of K1, the Zambian strains were clearly most closely related to each other and as a group were distinct from strains of types A, B, and C. Thus they were designated as Z strains. Nicholas *et al.* (1998) reported that HHV-8 genotyping based on K1 sequences is comparable to that obtained from sequences of ORF 26 and ORF 75. However, the K1-derived genotypes of some strains differed from their ORF 26 and ORF 75 genotypes, suggesting that the genotypes were not well resolved or that interstrain recombination has taken place between these widely spaced markers.

Using a quantitative assessment (likelihood mapping analysis) of the ability of a set of aligned sequences to yield robust phylogenetic information, we found that variation in the K1 gene allows the identification of well-resolved phylogenetic relationships among HHV-8 strains. This confirms and extends previous reports indicating that K1 is highly variable and useful for strain discrimination (Kasolo *et al.,* 1998; Nicholas *et al.,* 1998). In addition, we found that concatenation of two segments of nucleotide sequence totaling 246 bp that span the two most variable regions of K1, VR1 and VR2, allowed derivation of phylogenies essentially equivalent to

those obtained using nearly complete K1 sequences. This provides a rational basis for devising a more efficient typing system based on amplification and sequencing of these restricted regions. Such a system will be of use in larger-scale studies of HHV-8 transmission.

In this study, we identified a new HHV-8 genotype, designated genotype III, from Australia, that has not been recognized in other studies. However, samples from Australia or other Australasian locations have not been sequenced in other studies. The Australian strain classified in genotype III was phylogenetically distant and clearly distinct from the other genotypes. Additional strains will be needed to assess the prevalence of this genotype.

The strains studied here were classified into four genotypes and several subgroups were identified in one genotype. As more strains from more geographic locations are analyzed, genotype and subgroup boundaries and designations are likely to change, thus the designations used here must be considered provisional.

Geographic distribution

The data from this study and phylogenetic analyses of all currently available K1 sequences revealed previously undescribed trends in the geographical distribution of HHV-8. In contrast to the report from Zong *et al.* (1997) that a large cohort of US AIDS patients may have been infected by a single common strain (denoted type A), we found that individuals from the United States, regardless of their HIV or KS status, were infected by two genotypes of HHV-8, I and II, that correspond to types A and C, respectively, in the Zong nomenclature (Zong *et al.,* 1997). Although the HHV-8 strains in genotypes I and II are more closely related to each other than to strains of genotypes III or IV, the genotypes can be clearly distinguished. Among the Ugandan strains, we found only the less common genotype IV (type B of Zong *et al.,* 1997) and strains of subtype I-F. Furthermore, all strains reported by Kasolo *et al.* (1998) from Zambia, another central African country, clustered into subtype I-F.

All of the European strains analyzed in this and another study (Boralevi *et al.,* 1998) were of genotype II (type C of Zong *et al.,* 1997) suggesting that this genotype is predominant in Europe. In Australia and New Zealand, three HHV-8 genotypes were identified: the single representative of genotype III and genotypes I and II, which were also found in the United States. Although the data are scanty, we can speculate that genotype II originated in Europe; genotype III in Australasia, which has a mixed indigenous and migrant population; and genotype IV in Africa. The possible origin of genotype I, which is more widespread, is unclear. The biological significance of the apparently restricted distribution of HHV-8 genotypes remains to be determined.

Temporal and tissue distribution

In individuals with or without HIV infection, who had either KS or malignant lymphoma, no sequence variation was observed among HHV-8 strains derived from specimens obtained from different body sites and at multiple time points. This suggests that individuals are infected by a single predominant HHV-8 strain that is genetically stable. It raises the question as to how and when genotypic variation arises. In addition, the sequences we obtained from BCBL-1 and BC-1 cell lines that had been passaged in our laboratory for >1 year were identical (other than for a single nucleotide difference in a noncoding region of BCBL-1) to those published by others, further supporting the concept of strain stability. Our results are in agreement with those of Nicholas *et al.* (1997), who also found no genomic differences among multiple KS lesions obtained from the same patient or among subcloned PEL cell lines with different passage history from the same tumor, such as HBL6 and BC-1. These results differ from the observations of Di Alberti *et al.* (1997), who reported that in sarcoidosis patients, different HHV-8 ORF 26 sequences were identified in specimens collected from different lymph nodes in the same individual. The differences could be due to a variety of reasons, such as analysis of different gene segments (K1, gB, and gH versus ORF 26), the use of DNA polymerases with different error rates (*Pfu* versus *Taq*), and the analysis of specimens from different combination of body sites and diseases (KS biopsy, effusion, and PBMCs versus lymph nodes; AIDS KS and HIV-seronegative lymphoma versus sarcoidosis).

Lack of genotype–disease associations

Although some groups have identified possible links between genotype and particular diseases (Luppi *et al.,* 1997; Boralevi *et al.,* 1998), others have not (Kasolo *et al.,* 1998). We also were unable to identify any such links. However, the number and sources of specimens collected for this study were insufficient to rigorously investigate genotype–disease associations. The methods described here will be useful in future studies on this issue.

We were unable to amplify K1 fragments from any HHV-8 strain with group b gBN sequences; this resulted in a number of undetermined HHV-8 genotypes. This is not likely to have been due to a technical problem because the K1 unamplifiable material represented a variety of cellular sources from two continents and sequences were obtained from one or two other markers for each of these templates. It is possible that these strains encode a K1 sequence that is not able to be amplified by the primer set we used and that they possibly belong to an additional HHV-8 subtype or genotype. Resolution of this will require further study.

In summary, we have developed a DNA sequence-

based system for HHV-8 strain differentiation and genotyping and have shown that HHV-8 falls into at least four distinguishable genotypes (I–IV), one of which contains a number of subtypes. This system provides a powerful tool for studying HHV-8 transmission and disease associations.

MATERIALS AND METHODS

Clinical samples

Specimens from which HHV-8-specific sequences were derived for primary phylogenetic analyses are listed in Table 1; they included KS skin lesion biopsies, cultured KS lesion biopsies, PBMCs, lymph nodes, pleural effusions, and previously described pleural effusion-derived cell lines from 38 HIV-positive and 4 HIV-negative individuals. Of those individuals, 31 had KS, 2 had both KS and PEL, 6 had lymphoproliferative disorders or malignant lymphoma, and 1 was a healthy blood donor; 26 were from the United States, 8 were from Australia, 1 was from New Zealand, 1 was from Germany, 4 were from Uganda, and 2 were from Zambia. In some cases, more than one specimen was collected from the same individual, either from different body sites (six individuals), including PBMCs and skin, or at multiple time points (two individuals).

Cell lines and culture of KS biopsies

BC-1 cells (Cesarman *et al.,* 1995b) were cultured in RPMI 1640 medium with 20% FBS, 1% penicillin/streptomycin, and 1% glutamine. BCBL-1 cells (Renne *et al.,* 1996) were cultured in RPMI 1640 medium with 10% FBS, 1% penicillin/streptomycin, 1% glutamine, and 50 μM 2-mercaptoethanol. KS biopsies were minced and cultured in RPMI 1640 medium with 10% FBS, 1% penicillin/streptomycin, 1% glutamine, 10,000 U/ml heparin, and 50 μg/ml Endothelial Mitogen (ICN Biomedical, Irving, CA) in porcine skin gelatin-coated flasks (Yang *et al.,* 1994).

Specimen preparation

For US specimens, PBMCs were separated from fresh heparinized blood on Ficoll–Hypaque. The cells were washed twice with PBS, pelleted, and then stored at −70°C until DNA isolation. Some PBMCs were resuspended in freezing medium (DMSO) and stored directly in liquid nitrogen for several years before the DNA isolation. In these cases, the thawed PBMCs were centrifuged at 16,000 *g* for 15 min. DNA was isolated from both the cell pellet and supernatant. For Australian specimens, PBMCs were obtained by Ficoll–Hypaque, then mixed with freezing medium and stored in liquid nitrogen until DNA isolation. For Ugandan specimens, heparinized peripheral blood was mixed with Glycigel (Kaye *et al.,* 1991) and PBMCs were obtained by Ficoll–Hypaque. KS tissues from U.S. patients were obtained by punch biopsy. The tissues were immediately placed in 0.9% NaCl solution at 4°C and transported to the laboratory within a few hours. Tissue specimens were minced and stored at −70°C until DNA isolation. Some KS biopsy specimens were cultured for one or two passages before cells were collected for DNA isolation. Lymph nodes from Australian patients 1 and 8 were ground in PBS and then stored at −70°C until DNA isolation. A lymph node from Australian patient 5 and KS biopsies from Zambian patients were paraffin embedded. Pleural effusions were centrifuged at 13,000–16,000 *g* for 5 min. DNA was isolated from both the cell pellet and the supernatant.

DNA isolation

Genomic DNA was isolated from PBMCs of both US and Australian patients, cultured KS biopsies, ground lymph nodes, and pleural effusions (Easy-DNA kit; InVitrogen, Carlsbad, CA). Total lysates of KS biopsies from US patients were prepared by incubation overnight in lysis buffer (1% 10-lauryl ether, 10 mM Tris, pH 8.5, and 200 μg/ml proteinase K). KS tissue blocks from Zambian patients were sectioned and deparaffinized using xylene and ethanol. The DNA was then purified by phenol–chloroform extraction and ethanol precipitation and then suspended in water. PBMCs from Ugandan patients were washed twice with lysis buffer (10 mM Tris–HCl, pH 7.5, Triton X-100, 0.31 M sucrose, and 5 mM $MgCl_2$). The nuclear pellet was then incubated overnight in lysis buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% Tween 20, 0.45% Nonidet P-40, and 50 μg/ml proteinase K).

PCR amplification

Four gene fragments [K1 (886 bp), gBN (383 bp), gBC (534 bp), and gHM (716 bp)] from three HHV-8 genes were amplified by nested PCR using the PCR primers shown in Table 4. First-round amplification of 0.1–0.5 μg of genomic DNA or 2 μl of cell lysate was performed in a 50-μl reaction with each set of outer primers. Second-round amplification of 5–10 μl of the first-round reaction mix was performed in a 100-μl reaction with each set of inner primers. Reaction mixtures contained 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg/ml nuclease-free BSA, 200 μM concentration of each dNTP, 2.5 U of cloned *Pfu* DNA polymerase (Stratagene, San Diego, CA), and 0.5–1 μM concentration of each primer. Cycling parameters are shown in Table 5 for each gene segment. PCR started with the first cycle at 92°C for 2 min, followed by 5 cycles of denature at 94°C for 30 s, anneal A, and elongate A, and then 25 cycles of denature at 94°C for 30 s, anneal B, and elongate B, followed by the final cycle at 68°C for 7 min. PCR (8 μl) product from the secondary

TABLE 4

Primers for PCR Amplification of HHV-8 Genes

Gene	PCR round	Primer	Genomic coordinates[a]
K1	1st	GGCCCTTGTGTAAACCTGT	51–69
		AGTATCCGACCTCATAAAATG	1081–1061
	2nd	GACCTTGTTGGACATCCTGTA	76–96
		ACTGGTTGCGTATAGTCTTCC	961–941
gBN	1st	GACCTGTACGCCCTTCTGTAC	8653–8673
		ATAGAGTGGCACGGGTCTC	9136–9118
	2nd	GCCACCCTGGGGACTGTCAT	8720–8739
		TTGGTGATGGCGGACTCTGTC	9102–9082
gBC	1st	CCTGGGTGGCATCGGAAAAAC	10795–10815
		GCGTGGGTTGCCTCACAGTGT	10432–11413
	2nd	ATTGGTTACCGGATTCATAAA	10858–10878
		GGGTCGATAAATGGATTGA	11391–11373
gHM	1st	GCGCTCTATGGAGTGGTGTC	37689–37708
		CTAGAAAGCAGGGGGAGAATG	38624–38605
	2nd	GACGGCGTCCCATCTTCTGTT	37760–37781
		GGGCAGCTGTCGGTGAGG	38473–38456

[a] Coordinates are from GenBank accession number U75698 (Russo *et al.,* 1996).

amplification was electrophoresed in 1 or 1.5% agarose gels. As described in detail elsewhere (Spira *et al.,* manuscript in preparation), a strict protocol was followed for contamination control. Briefly, DNA template preparation was done in a laboratory in a different building from the PCR. PCR was conducted in dedicated rooms with dedicated equipment: a positive air pressure clean room for reagent preparation, a low-titer PCR assembly room for nonamplified clinical specimens, a high-titer PCR assembly room for cell line-derived templates, a thermocycler room, and a room for handling post-PCR procedures, such as gel electrophoresis. PCR work stations were irradiated for at least 8 h at 40 W/cm^2 (station for reagent preparation) or 100 W/cm^2 (station for PCR reaction assembly) by mercury vapor UV lamps after each use. Nonporous surfaces within the labs were wiped down regularly with 10% Clorox and then 70% ethanol. New reagent lots were evaluated before their use. Appropriate negative and positive control specimens were included in each set of PCRs. The quantity of specimens from the two Zambian patients was limited, and PCR amplification was done only for the gBC segment.

Direct PCR amplification from paraffin-embedded lymph node

A thin section of paraffin-embedded lymph node was placed in a 0.5-ml microcentrifuge tube, submerged in 25 μl of 1% Triton X-100 in water, and heated at 95°C for 15–20 min (Burns *et al.,* 1997). First-round amplification of 1-, 2.5-, and 5-μl aliquots of preheated sections were performed in 50-μl reactions as described above. Second-round amplification and product detection were as described above. At least two sections were tested for each gene fragment.

DNA sequencing

Amplimers were purified for sequencing (QIAquick PCR purification kit or QIAEX gel extraction kit; Qiagen, Inc., Chatsworth, CA) and then suspended in water. The purity and quantity of the DNA were determined by agarose gel electrophoresis and by fluorometry (DyNA Quant 200; Hoefer Pharmacia Biotech Inc., San Francisco, CA). An aliquot (50 ng) was then directly sequenced using inner (second-round) primers for each gene, fluorescent-dye terminators, and *Taq* polymerase on an automated sequencer (ABI 377; Applied Biosystems, Inc., Foster City, CA). Additional primers were used for sequencing the K1 and gHM gene fragments. Both strands were sequenced in their entirety.

TABLE 5

PCR Amplification Conditions

Gene	PCR round	Anneal A		Elongate A		Anneal B		Elongate B[a]	
		°C	s	°C	s	°C	s	°C	s
K1	1st	49	30	68	120	51	30	68	120
	2nd	49	30	68	120	51	30	68	120
gBN	1st	54	30	68	60	56	20	68	60
	2nd	58.5	30	68	45	59.5	20	68	45
gBC	1st	56.5	30	68	80	58.5	20	68	75
	2nd	54.5	30	68	65	55.5	20	68	60
gHM	1st	55	30	68	110	57	20	68	105
	2nd	58.5	30	68	85	59.5	20	68	85

[a] Increase elongation time 10 s/cycle.

Alignments and phylogenetic analysis

DNA sequences of amplified products, excluding the primer regions, were assembled with GelAssemble (GCG, Madison, WI). Alignments were performed with the Pileup and Lineup programs of GCG and Clustal W (Thompson *et al.,* 1997). For some analyses, amino acid sequences from the K1 VR1 and VR2 regions (Nicholas *et al.,* 1998) were concatenated and designated as K1 VR1VR2. The K1 VR1VR2 sequences were aligned and regions in the alignment that contained gaps were deleted before analysis. To evaluate the suitability of the data for phylogenetic reconstruction, likelihood mapping analysis (Strimmer and von Haeseler, 1997) was performed using PUZZLE 4.0. Maximum-likelihood tree construction for phylogenetic relationships was done by quartet puzzling (Strimmer and von Haeseler, 1996; Strimmer *et al.,* 1997) with PUZZLE 4.0 and the PHYLIP package (University of Washington, Seattle, WA).

Nucleotide sequence accession numbers

The DNA sequence data from the genotype I-A (US 3), I-F (Ug 3), II (US 6), III (Au 1), and IV (Ug 1) of K1 genes are available from GenBank (accession numbers AF151688, AF151690, AF151686, AF151687, and AF151689).

Addendum

After this manuscript was submitted for publication, Zong *et al.* published a paper [Zong, J. C., Ciufo, D. M., Alcendor, D. J., Wan, X., Nicholas, J., Browning, P. J., Rady, P. L., Tyring, S. K., Orenstein, J. M., Rabkin, C. S., Su, I. J., Powell, K. F., Croxson, M., Foreman, K. E., Nickoloff, B. J., Alkan, S., and Hayward, G. S. (1999). High-level variability in the ORF-K1 membrane protein gene at the left end of the Kaposi's sarcoma-associated herpesvirus genome defines four major virus subtypes and multiple variants or clades in different human populations. *J. Virol.* **73,** 4156–4170] describing high variability in the HHV-8 K1 gene and the identification of four major HHV-8 genotypes. The four major groups they identified correspond to those identified in this work, and the broad patterns of genotype geographic distribution are similar to those described here. Thus elements of this work confirm their result regarding geographic distribution of HHV-8 genotypes and extend the results to other countries and populations. Our genotypes I, II, III, and IV correspond to the HHV-8 subtypes A, C, D, and B, respectively, of Zong *et al.* Insufficient information is available to unify the description of groups below this level.

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